OPTIMIZATION OF BIOPOLYMER PRODUCTION IN SHAKE FLASK USING OIL PALM TRUNK SAP MEDIUM

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ABSTRACT

Polyhydroxybutyrate (PHB) is a biodegrable polymer which is an intracellular product produce by a bacteria. This research is conducted to optimize the biopolymer production in shake flask using oil palm trunk sap medium with *Cupriavidus necator.* It consists of four major phases starting with the innoculum development process, followed by the fermentation process, the analysis of the process and the process optimization. The innoculum development process is done to produce the 50 ml of innoculum *Cupriavidus necator* in shake flask by using growth media and incubated in incubator shaker with set point values for agitation speed and temperature. This experiment is followed with the fermentation process involving the manipulation of the parameters agitation speed, temperature and percent volume of oil palm trunk sap. Among the experimental points, it is found that the highest yield of PHB is produced in conditions of 150 RPM agitation speed, 32^{o}C temperature and 20% volume of oil palm trunk sap. This experiment was followed by mathematical analysis using Yates' Method to study the main effects and interactive effects of changing the level of experimental variables. The calculation showed that percent volume of oil palm trunk sap has a significant main effect on PHB production while the significant interactive effect is the combination of agitation speed and percent volume of oil palm trunk sap . From the experiment for Method of the Path of Steepest Ascent the highest production of PHB occured at parameter 31.1 ^{o}C for temperature, 138 RPM for agitation speed and 33.8% for percent volume of oil palm trunk sap.

ABSTRAK

Polyhydroxybutyrate (PHB) adalah plastik yang boleh dihuraikan dan ia dihasilkan di dalam sel bakteria. Penyelidikan ini adalah untuk menghasilkan bioplastik yang optimum dalam kelalang menggunakan jus batang pokok kelapa sawit dengan *Cupriavidus necator*. Ia mengandungi empat fasa yang dimulai dengan pembiakan bakteria, diikuti dengan proses penampaian, proses analisis and analisis mathematik. Proses pembiakan bacteria dilakukan untuk menghasilkan 50 ml bacteria *Cupriavidus necator* dalm kelalang menngunakan media pembesaran and diletakkan didalam incubator dengan temperature dan kadar kelajuan udara yang telah ditetapkan. Kemudian, eksperimen dietruskan dengan proses penampaian yang melibatkan manipulasi pembolehubah suhu, kadar kelajuan udara and peratus kandungan jus batang kelapa sawit. Daripada kesemua proses eksperiment, didapati bahawa PHB paling banyak dihasilkan dalm persekitaran 150 RPM kelajuan pengadukan, 32^{o}C suhu and 20% peratus kandungan jus batang kelapa sawit. Eksperiment diteruskan dengan analisis matematik menggunakan teknik Yates' untuk mengkaji kesan utama dan kesan saling tindak akibat perubahan aras pembolehubah eksperiment. Pengiraan menunjukkan bahawa peratus kandungan jus batang kelapa sawit mempunyai kesan utama terbesar terhadap penghasilan PHB manakala kesan saling tindak terbesar pula adalah daripada kombinasi kelajuan pengadukan dan peratus kandungan jus batang kelapa sawit. Daripada pengiraan dan eksperimen menggunakan teknik Steepest Ascent, parameter yang menghasilkan kadar PHB yang paling banyak ialah 31.1 ^{o}C suhu, 138 RPM kelajuan pengadukan dan 33.8% peratus kandungan jus batang kelapa sawit.

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LIST OF SYMBOLS

PHB	- Poly- β-hydratebutyrate
PHA	- Poly-β-hydroxyalkonoate
RPM	- Rotation Per Minutes
T	- Temperature
%	- Percent
%v/v	- Percent volume over volume
g	- gram
L	- Liter
M	- Molar
OD	- Optical density
SPSS	-Statistical Package for Science Society

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CHAPTER 1

INTRODUCTION

1.1 Background Study

Polyethylene or known as a plastic or polymer has been develop since 1936 by the American, British and German companies. Until today, plastic has become widely used material in our life due to its function and application in daily life such as packaging, cutlery, stationary and more. These phenomenons happen because the plastic is cheap, water resistant, flexible, durable and chemically resistant.

Although plastic has advantage but it also have disadvantage. One of its disadvantages is it is not biodegradable and high resistance to the natural degradation process. This will be harmful to the environment and lead to the crucial problem to environment in space for landfill (Huisman et al.). This is because it cannot undergo the biodegradation process and it takes many years or even centuries to it is fully degraded. Biodegradation is the process by which organic substances are broken down by the enzymes produced by living organisms. This shows that the plastic is not environment friendly.

Therefore we must find other source that is more environmental friendly and biodegradable to settle this problem. The search of biodegradable plastic has led to many type of biopolymer. Amongst all, microbially-formed polyhydroxyalkanoates (PHAs) is most suitable for significant contributions as 'bioplastics' or biodegrable plastic. Poly-hydroxyalkanoates (PHA) have attracted a lot of attention recently as biodegradable thermoplastics, and poly-hydroxybutyrate (PHB) is one of PHA and it is the best known. PHB has been produced through bacterial fermentation, being synthesized under limited culture conditions, and it is usually produced through the use of microorganisms that belong to the genres Alcaligenes, Azobacter, Bacillus, and Pseudomonas. (D.Z. Bucci, 2007). These biopolymers have similar properties to some of petrochemical-derived thermoplastics such as polyethylene in term of molecular weight, melting point, stiffness, brittleness and glass transition temperature (Steinbuchel & Funchtenbush, 2003). The polyhydrobutyrate (PHB) has been confirmed as an energy and carbon source in storage in various bacteria apparently in response to conditions of physiological stress (Kim et al., 1994). PHB is a biopolymer which is produced as a biodegrable intracellular microbial thermoplastic that provides carbon and energy reserves in the microorganism. Production of PHB is very expensive, so that this study is to optimize the production of PHB and reduce the cost of production.

PHB is produce by using the glucose as a food sources to the microorganism but the use of glucose is very expensive, so that instead of using the glucose, this research is use the oil palm trunk sap as a food sources to the microorganism. Oil palm trunk sap has a high content of glucose and suitable to grow the microorganism and fermentation process to produce PHB (Kosugi et al., 2010). This will reduce the cost of material for PHB production.

1.2 Problem Statement

PHB is produce through the fermentation in shake flask using the oil palm trunk sap medium. The cost to produce the PHB is usually expensive because there step that use the microbial. The biopolymer is extract from the inside of the microbial which make it expensive. It also need the sterilisation to avoid the contamination from other transmissible agents such as fungi, bacteria and spore forms at a surface of equipment and biological culture medium which is will affect the product. Besides that, the use of glucose as a food source to the microorganism in production of PHB is also expensive. So that, we need to optimize the production of PHB to reduce the cost using an inexpensive material as a food sources to the microorganism and it is also to reduce the use of polymer which is harmful to the environment.

1.3 Research Objective

The objective of this research is to optimize the production of biopolymer (PHB) in shake flask fermentation using oil palm trunk sap medium by manipulating the parameters which are agitation speed, temperature and percent volume of oil palm trunk sap.

1.4 Research Scopes

1. Obtaining from literature for suitable media variable.
2. Running the fermentation in shake flask at agitation speed, temperature and percent volume of oil palm trunk sap medium using Cupriavidus nectator.
3. Determining the analysis of dry cell weight and PHB.
4. Optimization of the variables which are agitation speed, temperature and percent volume of oil palm trunk sap medium in shake flask for maximum value
5. Determining the mathematical analysis to study the effect of variables on production of biopolymer in the shake flask fermentation.

The media variables that use in this research are

- Agitation speed (150 rpm to 250 rpm)
- Temperature (28 ^{o}C to 32 ^{o}C)
- Percent volume of oil palm trunk sap medium (10% to 20%)

1.5 Rational and Significant of the Research

This research is to lower the cost of PHB production and optimize the PHB produce in the shake flask fermentation by using inexpensive material as a food source to the microorganism.

CHAPTER 2

LITERATURE REVIEW

2.1 Development of Biopolymer Production

Together with the world which is full with a modern technology and new inventions to fulfil the human needs, one group of materials is found and produce. This material is known as synthetic polymer or plastics. This type of material is been widely used in daily usage to improve the lifestyle around the world. But, the used of too many polymers give a negative effect to the environment due to high resistance to natural degradation. Statistically, the generation of solid waste per capita in Malaysia which is been produce is 0.45 to 1.44 kg/day and most of the waste is consist of polymer based material. There are 177 disposal sites in Peninsular Malaysia is needed to dispose the solid waste through the landfill method (Malaysia Country Report, 2001).

Malaysia has faced the limitation of landfill problem to dispose the solid waste because of the usage of the plastic, polyethylene based material which is non-biodegradable. Biodegradation is a process of chemical breakdown by a physiologically environment where the materials are degraded aerobically with oxygen or anaerobically without oxygen. Sometimes, this process is related to the environmental remediation or bioremediation (Diaz, 2008).

To overcome this problem, the production of polymer which is can degrade by natural degradation is needed. The type of polymer which is can degrade by the natural degradation is biopolymer or bioplastics. The production of the biopolymer is important due to its biodegradable property compare to the conventional type and did not give negative effect to the environment. Biodegradable matter is an organic material such as plant and animal or other substances originated from living organisms or artificial that similar enough to plant and animal matter to be put to use and degrade by microorganisms (Diaz, 2008). One of the biopolymer that has been invented is polyhydroxybutyrate (PHB).

2.2 Polyhydroxybutyrate (PHB)

2.2.1 History of Polyhydroxybutyrate (PHB)

Polyhydroxybutyrate (PHB) is a polymer or family of PHAs that was first isolated and characterized in 1926 by French microbiologist Maurice Lemoigne as a constituent of bacterium Bacillus megaterium . Since then PHB has been shown to occur in a variety of taxonomically different groups. Most of the organisms (e.g. Azotobacter, Bacillus, Pseudomonas, Rhizobium, Methylotroph, etc.) are capable of accumulating PHB up to 30–80% of their cellular dry weight. PHB is produced by microorganisms apparently in response to conditions of physiological stress. The polymer is a primarily product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA (Poirier et al,1995).

2.2.2 Introduction of Polyhydroxybutyrate (PHB)

Poly-b-hydroxybutyrate (PHB) is an intracellular storage compound that provides a reserve of carbon and energy in several microorganisms (Anderson et al, 1990). Both prokaryotes and eukaryotes have been producing it although its accumulation is considered only in some prokaryotes. It accumulates as distinct inclusions in the cell and comprises up to 80% of cell dry weight for strains of Ralstonia eutropha also known as Cupriavidus necator, under conditions of nitrogen or phosphate limitation and excess of carbon source(Shimizu et al, 1992) The polymer was first recognised and characterised by Lemoigne in 1925 (Gross et al, 1988). The structure of PHB is shown in the Figure 2.1

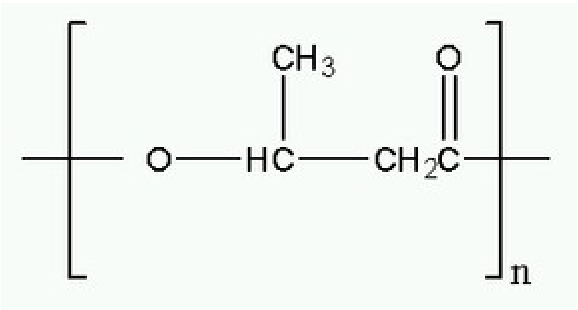

Figure 2.1: Structure of PHB

PHB is biodegradable polymer, which accumulates in the form of intracellular granules by a large variety of bacteria. Production of organic polymeric materials is currently one of the principal areas of PHB is a thermoplastic material that has attracted much attention due to such properties as biocompatibility and biodegradability. Microorganisms in nature are able to degrade PHa using their enzymes such as PHA hydrolase and PHA depolymerases (Jendrossek and Handrick, 2002; Choi et al, 2004). These granules act as energy reserve materials when nutrients such as nitrogen and phosphorus source are available in limiting concentration in the presence of excess carbon source (Anderson et al, 1990).

2.2.3 Properties of Polyhyroxybutyrate (PHB)

PHB is a one of the type for PHA. It can be degraded by bacteria and also often used as a taxonomic characteristic. Cofeeding of the substrates will give the formation of polymer containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers to form the copolymer of the PHB (Brandl et al, 1988).

PHB has the similar characteristics to the polyethylene plastic such as flexible, water insoluble, easy to use and more. But, PHB is more advanced compare to the polyethylene plastics because it is biodegradable and nontoxic material. It also relatively resistance to hydrolytic degradation which is make it different from other polymer (Peijis, 2002). PHB has a biodegradable ability which is can make the degradation process happen by the nature. The degradation process is depending on the activities of enzyme in the microorganism. The process is depending on the composition of the polymer, physical form, dimension and also environmental conditions (Jendrossek et al., 1998).

After extraction process from the cell, PHB is in the crystalline state. So, at that state, it is stiff but brittle material (Doi, 1995). It has glass transition temperature of 4°C and melting temperature of 180°C respectively (Sudesh et al., 2002). The specific density is 1.25 g /cm3. It is moisture resistant, shows piezoelectric effect and above all is truly and completely biodegradable (Doi, Steinbuchel, 2002). PHB also has the tensile strength 40 MPa, which is close to polypropylene strength. Other physical property of PHB is sinking in water (while polypropylene floats), and cause facilitating its anaerobic biodegradation in sediments.

However, the PHB have some disadvantage due to its tendency to be brittle as the plastic material. When it was spun into fibres it behaves as a hard-elastic material (Antipov et al, 2006). Besides, the synthesis process of PHB is economically challenging compare to the production of polyethylene plastic.

2.2.4 Application of Polyhydroxybutyrate (PHB)

Polyhydroxybutyrate or PHB is a biodegradable plastic and it can replace the non-biodegradable plastic such as polyethylene based material. The use of the non-biodegradable plastic can harmful to the environment and may cause significant ecological problems because it cannot be degraded by the nature. Meanwhile, PHB is a biodegradable product, biocompatible thermoplastic and has similar physical properties to polypropylene. It has similar piezoelectric properties to natural bone and is optically active (all of its monomers are the d-isomer).

There are many research have been done to extending the physical properties of PHB and the PHA family of polymer by compounding and blending to provide a corresponding broad range of end-use application. For example packaging and coating items. Besides that, PHB is also can be use in the medical industry such as medicine; veterinary practice, and in agriculture due to its biodegradable ability.

2.2.5 Metabolic Pathways of PHB Synthesis

The biosynthetic pathway of PHB consists of three enzymatic reactions catalyzed by three enzymes. The first reaction is the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by b-ketoacylCoA thiolase (encoded by phbA). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by phbB). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHB polymerase, encoded by phbC. PHB is synthesized by the successive action of b-ketoacyl-CoA thiolase (phbA), acetoacetyl-CoA reductase (phbB) and PHB polymerase (phbC) in a three-step pathway. The genes of the phbCAB operon encode the three enzymes. The promoter (P) upstream of phbC transcribes the complete operon (phbCAB) (Daae et al., 1999).

2.3 Cupriavidus nectator

Cupriavidus necator (formerly Ralstonia eutropha) is a gram negative, non-spore forming bacillus that is able to survive and flourish in mill molar concentrations of heavy metals, and plays a vital role in the formation of gold, a metal highly toxic to most other microorganisms. This organism shared with members of the genus Alcaligenes, which is comprised multiple species, including *Alcaligenes faecalis* (the type species), *Alcaligenes xylosoxidans* and allied species (now all classified in the genus Achromobacter; Yabuuchi et al., 1998) and *Alcaligenes eutrophus* (first reclassified in the genus Ralstonia (Yabuuchi, 1995) and recently transferred again, to the novel genus Wautersia (Vaneechoutte, 2004)). These bacteria produce the polyhydroxybutyrate (PHB) as an intracellular product which is inside the cell.

2.4 Oil Palm Trunks Sap

Oil palm trunks sap is a sap squeezed from the old palm tree felled. There are 64 million to 80 million old palm trees will be felled every year in Malaysia and Indonesia for replanting, as approximately 142 oil palms are usually planted in one hectare (Husin, 2000). The palm trunk has two parts that is inner part and outer part. The outer part is used for plywood manufacturing because of the strong physical properties. But the inner part is discarded in large amount due to its weak physical properties. Meanwhile, it is known that palm sugar and palm wine which is produced from sap obtained by tapping the inflorescence of varieties of palm species (Dalibard, 1999). The inner part sap has a large quantity of high glucose content sap in the trunk which is approximately 86.9 % and the concentration of the glucose is 85.2 g/l. The pH of sap is approximately 5.0 and the specific gravity is 1.07. The inner sap also contains amino acids, organic acids, vitamins and minerals. This shows that the inner oil palm trunk sap has sufficient nutrients to support fermentation (Kosugi et al., 2010).

2.5 Factorial Experiments Method

Factorial experiments method has many types but the common type which is usually used in the research is 2^n factorial experiments which involve two levels (Cochran et al., 1957). This method is designed to know the effect of a number of experiment variables on the yield to be investigated. It gives the main effect and interactive effect from changing its value from the lower level to upper level among all the experiments. The main effect is defined as the average of the effect of changing its value from the lower level to the upper level among all experiments. It is derived by assuming that the experimental variable is an independent parameter and all variations in its effects are due to experimental only.The interactive effects between two or more experimental variables are calculated on the assumption that the experimental parameters are not independent but are in fact interacting between them.

2.6 Yates' Method

This mathematical method is used to analyze the main effect and interactive effects. This method also can indicate whether the yield response surface in the area of examined is curved or in curved, or whether it is flat or not, or whether it isincreasing or decreasing with relation to one or more experimental variables and which direction.

CHAPTER 3

METHODOLOGY

3.1 Research Background

This research is to optimize the PHB production in small scale fermentation process which is conducted in 500mL shake flask with 200mL of working volume by using microorganism *Cupriavidus necator* sp, formerly known as *Ralstonia eutropha* sp to produce PHB which is the biopolymer.

The research consists five phase of experimental and mathematical methodology which are:

i. Obtaining the literature for suitable media variables.

ii. Mathematical method of Factorial experiment.

iii. Fermentation process.

iv. Sample analysis.

v. Mathematical method for optimization process.

3.2 Materials

The materials used in this research are microorganism *Cupriavidus necator,* mineral agar medium for cultivation of microbe, growth medium and oil palm trunk sap as the glucose resource.

3.3 Previous Research

The journal show that the parameters that give highest effect in PHB production are agitation speed of impeller, temperature of fermentation and initial concentration of glucose while the initial concentration of peptone give low effect on the production of PHB and can be negligible compare to another parameters (Azmi, A.,2009). Therefore, this research is conducted by using the agitation speed of impeller, temperature of fermentation and percent volume of oil palm trunk sap as a glucose resources.

3.4 Mathematical Method of Factorial Experiment

This mathematical method of Factorial experiment is used to calculate or determine the number of experiments that should carried out in order to investigate the effect that has three parameters simultaneously. The mathematical equation that used in this method is shown as below:

Number of experiment = 2^n

Parameter that used in this research, $n = 3$

Therefore,

Number of experiment = 2^3

= 8

From the calculation above, the table below is constructed to show the combination of the parameter that used in this research:

Table 3.1: Experiment Table Constructed

Number	Temperature (^oC)	Agitation Speed (RPM)	Percent Volume of Oil Palm Trunk Sap (%)
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1

Table 3.2: The Plan of the Replication of the Centre Point